Chromosome Aberrations and Sister Chromatid Exchange after *in Vitro* Exposure of Human Lymphocyte to Methyl Isocyanate (MIC)

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Abstract— Methyl isocyanate is an intermediate chemical in the production of carbamate pesticides. It is reactive and hazardous to human health. The toxic effect of the compound was apparent in the Bhopal disaster, where methyl isocyanate and other gases were released from the underground reservoirs of Union Carbide India Limited (UCIL) factory, over a populated area on December 3, 1984. In this present study CA and SCE have been measured. Positive implications about clastogenic effects by MIC induced cytogenic alteration in the form of chromosomal aberration in in vitro human lymphocyte culture was recorded. In chromosomal aberration, mainly centromeric breaks were high after addition of MIC as compared to control. So this indicates that MIC specifically acts on centromeric region of human chromosome. Centromeric region mainly composed of heterochromatin. Hence, it can be concluded that MIC mainly affects heterochromatin region of chromosomes. Even Dicentric, Ring, Premature Separation of Centromere were also observed. While there was no change in the SCE frequency in the exposed group compared to controlled one. But the rate of cell division was little decreased in the exposed individuals. And therefore to further justify, additional in vitro and in vivo studies need to be performed so as to confirm these data and to discover the mechanism of the possible protective effects.

Key words: Methyl Isocyanate, Chromosomal Aberration, Sister Chromatid Exchange, *In Vitro* Human Lymphocyte Cultures

I. INTRODUCTION

Methyl Isocyanate (MIC) is an organic compound with the molecular formula C_2H_3NO. Synonyms are isocyanatomethane, methyl carbylamine, and MIC. Methyl isocyanate is an intermediate chemical in the production of carbamate pesticides (such as carbaryl, carbofuran, methomyl, and aldicarb). It has also been used in the production of rubbers and adhesives. As a highly toxic and irritating material, it is hazardous to human health, and was involved in the Bhopal disaster which killed nearly 8,000 people initially and approximately 20,000 - 30,000 people in total [1-3]. Methyl Isocyanate (MIC) is colorless, lachrymatory, flammable liquid, soluble in water and easily reacts with water [4]. Its molecular mass is 57.051 g/mol, physical state-solidified melt, melting point -45°C, boiling point 39.5°C, odor- pungent, pH-6 and color-yellow. It is unstable and reactive and hazardous to human health.

The stability of this chemical compound is extremely flammable. It can form explosion with air combination. Notably it has very low flash point and boiling point. This chemical is very harmful and dangerous when inhaled and swallowed and it is dangerous as well when it is contact with skin, it may bring to burn skin and eyes that will result into permanent damage in the eye. Methyl isocyanate can be irritable in the respiratory track and cause allergy like asthma. This chemical compound can also be dangerous in generating reproductive harm. To protect away from this harmful chemical, one should have good ventilation, use gloves and safety glasses. The market around the world for diisocyanate during the year of 2000 was about a million tonnes of 4.4 which is composed of 61.3% methylene diphenyl diisocyanate, 3.4% total of hexamethylen diisocyanate, the toluene was 34.1% and the total for various others was 1.2%.

The toxic effects of the compound was apparent in the Bhopal disaster, when around 42,000 kilograms (93,000 lb) of methyl isocyanate and other gases were released from the underground reservoirs of Union Carbide India Limited (UCIL) factory, over a populated area on December 3, 1984, immediately killing thousands and leading to the deaths of tens of thousands in subsequent weeks and months. Among human survivors following the methyl isocyanate (MIC) gas tragedy the major complaints have been related to deep-seated suffocation, terrible pain in breathing, and severe ocular irritations. Generally the genetic effects of any chemical compound can be measured by three major parameters, viz., Chromosomal Aberrations (CA), Sister Chromatid Exchanges (SCE) and Micronuclei. In this present study CA and SCE have been measured.

Chromosomal aberrations are missing extra or irregular portion of chromosomal DNA. It can be from an atypical number of chromosomes or a structural abnormality in one or more chromosomes. Sister chromatid exchange is the process wherein the two sister chromatids breaks and rejoin with one another physically switching positions on the chromosome. It is a sensitive indicator of chromosomal damage.

In order to assess the possible genetic effects of MIC, Goswami et al [5] used lymphocyte cultures and screened chromosomes by looking for chromosomal aberrations and sister chromatid exchange frequencies. These parameters are the good indicators of genetic damage in chromosomal DNA. The results were compared to two groups of controls (one group comprising persons present in the same house; the second group of persons were chosen from distant places, 20–50 km away from the incident). Chromosomal breaks have been observed in 10 out of 14 MIC-affected people (71.4%) studied while only 6 out of 28 (21.4%) controls had chromosomal breaks. Some MIC-exposed persons had chromatin bodies in addition to the normal 46 chromosomes. These observations suggest that chromosomal DNA has been damaged.

In the present study we aimed to understand the chromosomal aberrations frequency and sister chromatid exchange frequency after *in vitro* exposure of normal healthy human lymphocytes to MIC compound. In doing so, information would be gathered about effects of MIC on chromosomal DNA. This would help to better understand toxic effects of MIC. Further once chromosomal aberrations be found out after *in vitro* exposures and a correlation of *in vivo* studies is established, a clear body of information would

be generated to clarify the fuzzy picture of MIC toxicity under *in vivo* conditions.

II. METHODOLOGY

Whole blood lymphocyte cultures were set up following the method of Hungerford [6] with some modifications [7]. To 5 ml of RPMI 1640 HiKaryo medium, 50 µl of heparin, was added. About 0.6 ml of whole blood (collected in sodium heparinized tubes) was added to the above mixture and culture vials were incubated at 37^0 C for 72 hours. In SCE batches, the compound 5- BudR was added in a final concentration of 10 µg/ml of culture after 24 hours of the initiation of the culture. At 69^{th} hour of incubation the cultures of both the batches i.e. CA and SCE were treated with 0.1 ml colchicines so as to arrest mitotic division.

The cultures were terminated after 72 hours of incubation by centrifuging at 1500 rpm for 10 minutes. The supernatant was removed and the pellet was treated with 5 ml of 0.075 M KCl for 20-25 minutes at 37^0C temperatures. The cells were once again centrifuged at 1200 rpm for 10 min, supernatant was removed and pellet was now mixed in 3:1 Methanol: Acetic acid (Carnoy's fixative) for about 1 hour at 4^0C in the refrigerator. The cells were again centrifuged at 1200 rpm for 10 min, the supernatant was removed and cells were washed with fresh chilled 3 ml of fixative. These steps of washing the cells were repeated twice or thrice so as to obtain white pellet. Once these steps were finished the cells were suspended in small quantity of fixative so as to obtain uniform density of cells. The drops of this cell suspension were allowed to fall from a convenient height onto a pre-chilled sterile glass slide. The slides were then air dried by placing on a hot plate at 40-45 ^{0}C. All slides were immediately blind coded.

Air-dried slides prepared from CA batches were routinely stained in 4-7% Giemsa and observed under microscope. One hundred well spread metaphase plates were scored per culture for counting chromosomal aberrations. Metaphase plates containing less than 44 chromosomes were not considered. Both chromatid as well as chromosome type of aberrations were considered. Achromatic lesions, often being visual aberrations was not included in calculating the aberration frequencies.

Air dried slides from those 5- BudR treated cultures were allowed to dry for a day and then stained in Hoechst 33258 in dark. After half an hour, cover-slips were placed on those slides with a drop of Hoechst 33258 staining solution; the edges of slides were then sealed with paraffin wax and then were exposed under fluorescent light. After 24 hours of exposure, the wax was removed thoroughly and the slides were incubated in 2X SSC (Double Strength Sodium Saline Citrate) solutions at 60^oC temperatures for 1 hour in water-bath. Thereafter, those slides were then washed in distilled water and stained in 4-7% Giemsa staining solution for 3-4 minutes [8].

About 30 well spread second division metaphase (M2) plates were scored for calculating SCE frequencies. The percentages of the first metaphases (M1) as well as third metaphases (M3) were counted so as to calculate the Replicative Index (RI).

III. WORKING PROTOCOL

Total of 12 human blood lymphocytes cultures were set up in enough sterile condition. Four different culture vials were set up per individual blood sample. The first vial was kept untreated so as to act as control for CA; the second vial was treated with MIC for CA. The third vial was treated with 5BudR for SCE while the fourth vial was treated with 5BudR along with MIC for SCE. The dose and concentration of MIC was fixed on the basis of pilot experiments. All these vials were then allowed to be incubated for 72 hours. All cultures were then terminated at the end of 72 hours. Slides were prepared from those vials and chromosomal aberrations and SCE frequency were compared with each other.

IV. RESULTS AND DISCUSSION

The present study was initiated to rule out the possibility of any kind of aberration and SCE due to Methyl Isocyanate against untreated *in vitro* cultured human lymphocyte chromosomes. There was an increase in total number of chromosomal aberrations (Table I) and non significant increase in SCE (Table II) after addition of MIC (10 µg/ml) as compared with untreated culture.

Fig 1: Normal human metaphase plate

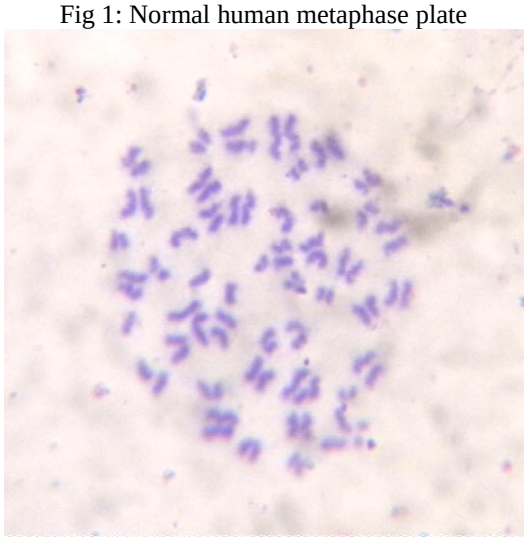

Fig 2: Premature Separation of Centromere

In chromosomal aberration, mainly centromeric breaks were high after addition of MIC as compared to control. So this indicates that MIC specifically acts on centromeric region of human chromosome. Centromeric region mainly composed of heterochromatin. Hence, it can be concluded that MIC mainly affects heterochromatin region of chromosomes. Even Dicentric, Ring, Premature Separation of Centromere were also observed. This indicates the hazardous nature and strong clastogenic potential of MIC. Plate I (Fig 1) indicates normal Giemsa stained human metaphase chromosomes. Plate I (Fig 2) indicates Premature Separation of Centromere. Dicentric chromosomes are shown in Plate I (Fig 3). Plate I (Fig 4) indicates Centromeric Break

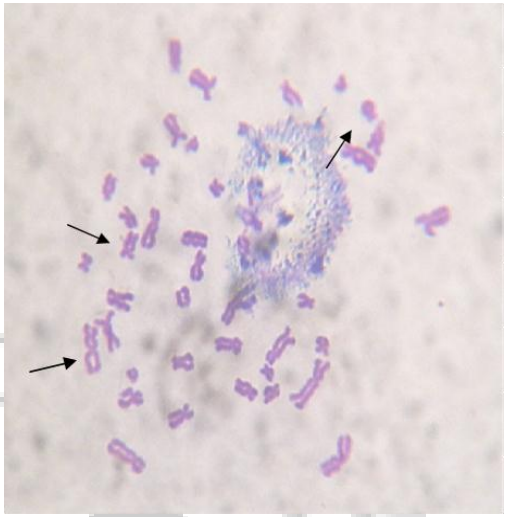

Fig. 3: Dicentric

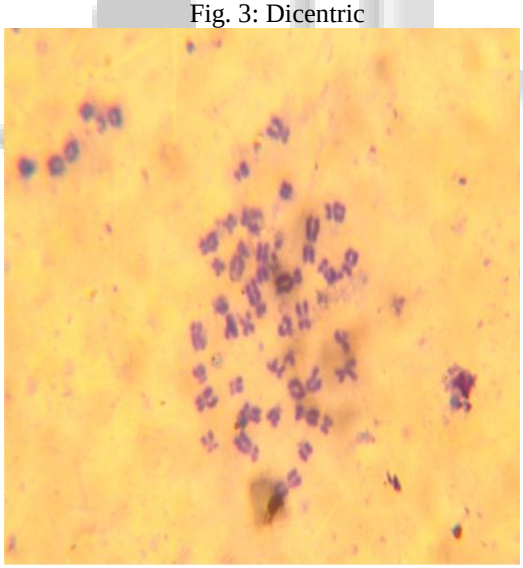

Fig. 4: Centromeric Break

Sister chromatid exchanges were found to occur in same frequency in the exposed group as compared to the control. In general, the number of SCE per cell was same in MIC exposed individuals as compared to unexposed group. The replicative index was little altered although non-significantly between the exposed and unexposed blood samples to MIC to the concentration of 10µg/ml. This reveals that the rate of cell division slightly decrease in the vials exposed to MIC.

Sr No	Aberration	Groups	
		Control	MIC (10µg / ml)
1	Chromatid Gap	02	04
2	Chromatid Break	01	08
3	Chromosome Gap	_	01
4	Chromosome Break	_	01
5	Centromeric Break	_	67*
6	Dicentric	_	06
7	Ring	_	01
8	Telomeric Association	02	03
9	Premature Separation of Centromere	–	04
10	Chromatid Interchange	03	01
11	Total aberration	08	96*

*significantly higher than the control at P<0.001

Table 1: Chromosomal aberrations induced in *in vitro* cultured human lymphocytes after addition of MIC. (Figures indicate total aberrations per 100 cells)

Subjects	No. of Individuals	No. of Metaphase Cells scored	SCE frequency and Replicative index				
			Percentage of cells			Mean SCE/cell	Replicative index
			M 1	M 2	M 3		
Control	12	1200	43	27	30	3.23	1.96
Methyl isocyanate (10µg / ml)	12	1200	52	32	16	4.76	1.62

M1- First divison Metaphase plate, M2- Second division Metaphase plate, M3- Third division Metaphase plate

Table 2: Sister Chromatid Exchange among *in vitro* cultured human lymphocytes after addition of MIC (Figure indicates mean SCE per 100 cells)

Isocyanates one of the highly reactive industrial intermediates, possess the capability to modulate the bio-molecules by forming toxic metabolites and adducts which may cause adverse health effects. Some of their toxic degradations have previously been unknown and overlooked; of which, molecular repercussions underlying their genetic hazards upon occupational/accidental exposures still remain as an intricate issue and are hitherto unknown [9]. Methylisocyanate (MIC) is prepared industrially by reacting methylamine with phosgene, oxidizing mono methyl formamide at high temperatures (≥550^{o}C), or heating metal methyl isocyanates [10] (Dave 1985). Because of its high reactivity, MIC is an intermediate in manufacturing of polyurethane foam, carbamate pesticides, herbicides and plastics. Tobacco smoke from some brands of cigarettes also contains MIC.

There are many investigations which proved the potential of MIC as a clastinogen. As a carbamoylating agent (R-N=C=O), MIC might be expected to react with nucleophilic sites on cellular macromolecules including Proteins, RNA and DNA. MIC may exert its genotoxicity by binding to nuclear proteins rather than by binding to DNA [11]. One report [12] was found on the direct interaction between nucleic acid bases and isocyanates, where carbamoylation of both endocyclic and exocyclic nitrogens were reported.

A case can be made that the genetic toxicity of MIC reported in this paper might be due in whole or in part to MIC disruption of the integrity of eukaryotic chromosomes through interaction with nuclear proteins. Although speculative, this possibility is supported by reports that alkylnitrosoureas lead to carbamoylation of the chromatin [13] (Sukhakar et al, 1970), and the nuclear matrix [14] (Tew et al, 1983).

In order to assess the possible genetic effects Goswami et al, [5] have used lymphocyte cultures and screened chromosomes by two techniques; one by looking for chromosomal aberrations and the other by estimating SCE frequencies. Both these parameters are good indicators of genetic damage in chromosomal DNA. SCE frequencies in lymphocytes have been increased more than three times in MIC-exposed persons. The results were compared to two groups of control; Sister Chromatid exchanges and CAs were found to occur in statistically high frequencies in the exposed batch as compare to the control.

The chromosome-type and chromatid-type aberrations that include dicentric chromosomes, double minutes, acentric fragments, ring chromosomes, chromatid breaks and terminal deletions, which were found to be statistically higher in the MIC exposed group ($p<0.01$) than the non – exposed [15]. An initial study [5] revealed chromosomal aberrations in 71.4% of the gas affected people of Bhopal. The carcinogenicity prediction and battery selection method was used to predict the probability of carcinogenicity of MIC. That analysis predicts that MIC has a significant potential for inducing cancer in rodents. The realization of the identified risk would be dependent upon level, duration, and mode of exposure [16]. The higher frequency of chromosomal aberrations may play a definitive role in the pathway of cancer progression and other genetic diseases [15]

The present study was carried out on *in vitro* cultured human lymphocytes. The cultures were treated with MIC. Positive implications about clastogenic effects by MIC induced cytogenic alteration in the form of chromosomal aberration in *in vitro* human lymphocyte culture was recorded.

In conclusion, the evidences provided in the current study give reason to believe that the MIC posses a clastogenic potential. The present study support the hypothesis that i*n vitro* MIC exposure to cultured human lymphocytes produces chromosomal aberrations and mainly MIC acts on the centromeric region of chromosome. Similarly, the genotoxic effect of MIC was investigated [17] using chromosomal aberration assay on mouse bone marrow cells, which revealed the same result that MIC induce more number of chromosomal aberrations in the form of Centromeric Breaks. Cenromeres are generally composed of heterochromatin. Hence, it is concluded that MIC mainly targets heterochromatin regions of chromosomes. While there is no change in the SCE frequency in the exposed group compared to controlled one. But the rate of cell division is little decreased in the exposed individuals. And therefore to further justify, additional *in vitro* and *in vivo* studies need to be performed so as to confirm these data and to discover the mechanism of the possible protective effect.

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